



Mechanisms underlying *Eugenia mattsii* D. Legrand leaves extract, fractions and compounds induce relaxation of the aorta from normotensive and hypertensive rats

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Abstract

The present study aimed to verify the effect of methanolic extract, fractions, and phenolic compounds of *Eugenia mattsii* D. Legrand leaves on the aorta relaxation. Isometric tensions were measured on the aorta of normotensive (NTR) and spontaneously hypertensive rats (SHR). The results showed that both methanolic extracts of leaves and stems, as well as, fractions obtained from leaves were able to induce a concentration-dependent relaxation in both endothelium-intact and -denuded aortas. The methanolic extract of leaves (ME-leaves) was the most effective since the maximal relaxation ($\approx 83\%$) obtained was at the concentration of 300 $\mu\text{g/mL}$. As the endothelium-dependent relaxation was more significant, we investigated the mechanisms by which ME-leaves induced this effect. After the pretreatment with LNAME, ME-leaves-induced relaxation was decreased in the aorta of NTR and SHR. However, the pretreatment with methylene blue only reduced the relaxation in the aorta of NTR. Furthermore, pretreatment with ME-leaves decreased phenylephrine-induced contraction in preparation Ca^{2+} -free only in aortic rings from NTR. This study also reveals that both compounds, cryptostrobin isolated from chloroform fraction and catechin from the ethyl acetate fraction induced a marked relaxation in endothelium-intact aortic rings of NTR. In conclusion, ME-leaves induces relaxation in the rat aorta involves the modulation of NO/cGMP dependent signaling pathway, this mechanism may at least, in part, explain the endothelium-dependent relaxation. Furthermore, cryptostrobin and catechin also induced relaxation, which may contribute synergistically to the vasorelaxation effect of the ME-leaves.

Keywords *Eugenia mattsii* · Vasorelaxation · Aorta ring · Nitric oxide · Hypertension

Introduction

The *Eugenia* genus is one of the largest within the Myrta-ceae family, presenting about 5000 species (Limberger et al. 2004; Fiuza et al. 2008). In Brazil, there are 350 native species from this genus (Landrum and Kawasaki 1997). *Eugenia* species are appreciated for their fruits such as *Eugenia uniflora* (pitanga), *Eugenia involucrate* (cereja or cherry) and *Eugenia caryophyllata* (cravo-da-índia). Other is used in folk medicine as antidiarrheic (*E. uniflora*) and antidiabetic (*Eugenia jambolana*) (Limberger et al. 2004;

Lopes et al. 2000). Previous studies demonstrate that the *Eugenia* genus is an important source of medicinal plants, that can present pharmacological promising activity for the treatment of several diseases, including cardiovascular diseases. Infusions or hot water extracts of *E. uniflora* are used as an antihypertensive and diuretic in traditional medicine (Amat and Yajjiá 1991; Bandoni et al. 1972; Ratera and Ratera 1980). Indeed, Consolini et al. (1999) found that aqueous extract of leaves of *E. uniflora* has a hypotensive effect mediated by direct vasodilatation and diuretic activity. However, the mechanism has not been investigated yet. Moreover, hydroalcoholic extracts of leaves from *E. uniflora* had a relaxation endothelium-dependent effect on aortic rings pre-contracted with noradrenaline. This vasodilatation was mediated by nitric oxide (Wazlawik et al. 1997). Besides, in one study performed on isolated heart of rats, *E. Jambolana* aqueous extract from leaves was able to exert cardiovascular activity (Consolini and Sarubbio 2002; Shukla et al. 2014).

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Recently Santos et al. (2018) demonstrate that methanolic extracts from *Eugenia mattosii* D. Legrand fruits reduced the injured areas against ethanol and indomethacin-induced gastric ulcer. It has been demonstrated that endogenous or exogenous substances that can maintain or increase gastric blood flow, due to their vasodilatory properties prevented the acute gastric injury (Magierowski et al. 2017; Farrugia and Szurszewski 2014).

Eugenia mattosii is a bush popularly known as “cerezinha de mattos” and despite the large distribution of this species, especially in Southern Brazil, the pharmacological profiles of this plant have not been investigated yet. For that, the purpose of this study was to demonstrate the effect and the mechanism of vasodilatation of the methanolic extract (ME) of *E. mattosii* dried leaves and stems, as well as their fractions and isolated compounds on aortic rings from normotensive and spontaneously hypertensive rats.

Many studies describe the potential of natural products for the treatment of cardiovascular diseases. Since blood pressure depends on the arterial tone and considering the therapeutic potential of the compounds described for the genus, as well as of others already studied species, we highlight the potential of the species *Eugenia mattosii* D. Legrand.

Arterial hypertension is a multifactorial disease characterized by increased blood pressure, arising from a complex interaction between genes and the environment. Even with unknown etiology, the study in an animal model of hypertension has shown to be an important tool for understanding the mechanisms that control blood pressure and allowing the development of new drugs for its treatment or prevention. Biological studies with animals are developed to understand changes in blood pressure and that several vascular beds are used. Although the aortic artery does not effectively participate in blood pressure control, the study in this vessel contributes to the compression of the cellular mechanisms involved in these pathologies. Besides, the use of the aorta artery allows the use of a reduced number of animals since from an aorta artery it is possible to use approximately eight rings.

Thus, based on these data and the wide distribution of the *Eugenia* genus in the south of Brazil and the use of several species by the population with their already confirmed pharmacological effects, we hypothesized that *E. mattosii* D. Legrand could have a vasodilatory effect.

Materials and methods

Animals

The experiments were performed with normotensive male Wistar rats (NTR: 200–250 g) and male spontaneously

hypertensive rats (SHR: 250–280 g). The animals were provided by The Central Animal Facility of Universidade do Vale do Itajaí (UNIVALI) and kept under conditions of constant temperature (22 ± 2 °C) with a 12 h light/12 h dark cycle with free accesses to water and food.

The studies and all methodologies used were approved by the Ethical Committee for the Care and the Use of animals of (UNIVALI) (Authorization No 055/17p), which adopted all the recommendations of the Guide for the Care and Use of Laboratory Animals and approved on November 10, 2017.

Plant material

The leaves and stems from *E. mattosii* were collected in the city of Itajaí-SC, Brazil.

The plant material was identified by Prof. Oscar Iza (Universidade do Vale do Itajaí, UNIVALI) and a voucher specimen was deposited at the Barbosa Rodrigues Herbarium under Number VCFilho 150, in Itajaí-SC, Brazil.

Preparation of extracts, fractions, isolation of compounds and phytochemical screening

Extracts, fractions, and compounds isolation and identification were provided by Vechi et al. (2019). Briefly, fresh leaves (560 g) and stems (700 g) of *E. mattosii* were minced and extracted separately by maceration with methanol at room temperature for 7 days. The solvent was filtered and concentrated in a rotary evaporator under reduced pressure and at a constant temperature (50 °C). After the elimination of methanol, the methanolic extract (ME-leaves 15.34% and ME-stems 2.75%), have been partitioned with solvents of increasing polarity (chloroform and ethyl acetate) to obtain the respective fractions and yields: chloroform fractions (CLF-leaves) (5.43%), CLF-stems (0.51%), ethyl acetate fractions (AEF-leaves) (2.77%) and AEF-stems (0.15%). Both fractions were submitted to a chromatographic column, separately, to isolate the major compounds. The chloroform leaf fraction (15 g) was submitted to open silica gel column chromatography (CC) eluted with hexane: acetone gradient, resulting in the collection of 183 subfractions (10 mL each). Similar subfractions were combined based on their thin layer chromatography (TLC) profiles, and re-chromatographed as before. Subfractions 75–89 (664 mg) furnished a pure solid, identified as pinostrobin (362 mg), while subfractions 153–177 (1056 mg) led to the isolation of 84 mg of cryptostrobin (Massaro et al. 2014; Solladié et al. 1999). The ethyl acetate fraction (12 g) was also submitted to chromatographic procedures as indicated before, using chloroform: methanol gradient. About 138 subfractions were collected and combined according to similarity. Sub fractions 48–56 (233 mg) was re-chromatographed given catechin (19 mg). The compounds were identified based on

TLC and spectral data (NMR) in comparison with standard sample and literature, respectively (Fig. 1) (Rosandy et al. 2013; Moresco et al. 2016; Wishart et al. 2009). Qualitative phytochemical screening of the ME-leaves and stems was performed to evaluate the presence or absence of flavonoids, anthraquinones, alkaloids, saponins, tannins, and terpenoids according to the previously described method by Biavatti and Leite (2005).

Estimation of total phenolic and flavonoids content

The content of total phenols was determined using the Folin–Ciocalteu reagent. In brief, about 50, 100, 150 and 200 mg/mL of extracts of ME-leaves and stems were mixed with 5 mL of distilled water. After 2.5 mL of Folin–Ciocalteu reagent (1: 1 dilution) and 2.0 mL of sodium carbonate (7.5% w/v) were added to each sample. Besides, the Falcons' tubes were incubated at 45 °C for 15 min. The absorbance was determined at a 760 nm spectrophotometer. The total polyphenol concentration was calculated from a calibration curve, using tannic acid as a standard. Results were

expressed as tannic acid equivalents (TAE) in mg. The flavonoids content was evaluated using the method of Dowd. In this methodology, 500 μ L of aluminum chloride (AlCl_3), prepared in 2% of methanol were mixed with the same volume of extract solution (400–100 μ g/mL) or distilled water (for white). In this protocol, white was used as a negative control to assure us of the positive results. It does not contain extract. Quercitrin was used as a standard for the calibration curve. The total flavonoids quantity was expressed in mg quercitrin equivalent per gram of ME-leaves and-stems.

In vitro 2,2-Diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity assay

The reducing potential of the stable free radical DPPH was determined under absorbance at 517 nm. The reaction medium was composed of 750 μ L of extract or the compound solution at different concentrations (1, 10 and 100 μ g/mL) and 250 μ L DPPH methanol solution (10 μ g/mL). After 5 min, the decrease in absorbance was measured. Samples of ascorbic acid (AA: 50 μ g/mL) and 10% dimethyl sulfoxide

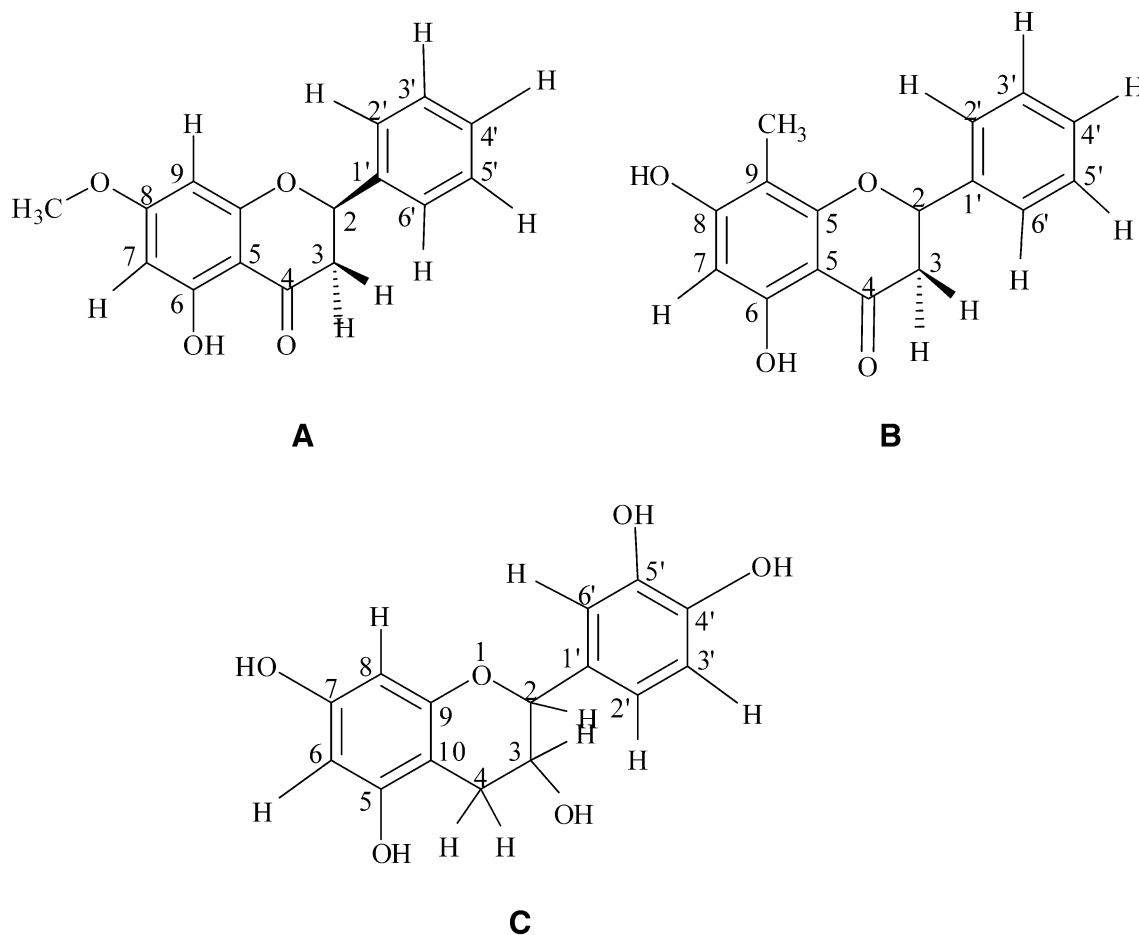


Fig. 1 Chemical structures of phenolic compounds pinostrobin (a), cryptostrobin (b) and catechin (c) isolated from *E. mattsosii* leaves

solution (vehicle) were used as a positive and negative control groups, respectively.

Drugs and reagents

Phenylephrine hydrochloride (PE), acetylcholine chloride (ACh), N^o-nitro-L-arginine methyl ester (L-NAME), indomethacin (Indo), atropine and methylene blue (MB) were purchased from Sigma (St. Louis, MO, USA). All drugs were freshly prepared in physiological saline solution (PSS). Methanolic extracts, chloroform, ethyl acetate fractions, and isolated compounds were prepared on the day of the experiment in regular Krebs's solution.

Preparation of rat thoracic aorta rings

After anesthesia with ketamine/xylazine (100/20 mg/kg) the thoracic aorta was quickly removed and placed in recipients containing Krebs's solution with the following composition (mM): pH 7.4; composition in mM: NaCl 115.3, KCl 4.9, CaCl₂·2H₂O 1.46, KH₂PO₄ 1.2, MgSO₄ 1.2, D-glucose 11.1, NaHCO₃ 25, cleaned of connective tissue and cut into rings (3–4 mm in length). In some preparations, the endothelium was removed by gently rolling the intimal surface of vessel lumen with a thin wire. The isolated aorta rings were then kept in organ baths containing 3 mL of Krebs's solution, under a resting tension of 1 g, maintained at 37 °C and continuously aerated with 95% O₂ and 5% CO₂. A 60 min equilibration period was applied before any experimental study, during which the bath was flushed with fresh Krebs's solution every 15 min. Following equilibration (60 min), rings were activated with KCl (60 mM) for 10 min to test their contractility. To confirm the presence of functional-endothelium a further 30 min stabilization period is expected for the addition of PE (1 μM) and, on the maximal contraction, the preparation was exposed to ACh (an endothelium-dependent vasodilator; 1 μM). Rings that produced > 80% relaxation to PE contraction, were considered having functional endothelium.

Pharmacological studies

Investigation of the vascular effect of ME, fractions and isolated compounds from *E. mattosii* in aortic rings from normotensive and SHR

In order to study the vascular effect of ME-leaves or stems, by chloroform (CLF) and ethyl acetate fractions (AEF) and phenolic compounds isolated from leaves, cryptostrobin and pinostrobin from the chloroform fraction and catechin from the ethyl acetate fraction, the aortic rings with and without functional endothelium were pre-contracted by phenylephrine

(an α₁-adrenergic receptor agonistic; 1 μM). During the tonic phase obtained with PE-induced contraction, the relaxation was evaluated through the addition of cumulative concentration of extracts, fractions (0.1–1000 μg/mL) and isolated compounds (0.1–300 μg/mL).

Evaluation of endothelium mediators and membrane receptors on the vascular effect of ME-leaves in aortic rings from normotensive rats

Comparing the results obtained in this study we found that ME-leaves were the most efficient to relax PE-contracted aortic rings. Therefore, the mechanisms that induce the vasodilatation were conducted using the ME from leaves. To investigate if NO/cGMP, prostacyclin (PGI₂) or muscarinic receptor were involved in the vasorelaxant effect, the endothelium-intact aortic rings were incubated with L-NAME (a nitric oxide synthase inhibitor; 100 μM), MB (a soluble guanylyl cyclase inhibitor; 100 μM), indomethacin (a non-selective inhibitor of cyclooxygenase; 10 μM) or atropine (a muscarinic receptor antagonist; 1 μM) for 30 min prior the addition of PE. ME-leaves were then cumulatively added to the baths.

Effects of ME-leaves on extracellular calcium influx and intracellular calcium store

To verify the involvement of calcium uptake or its intracellular release in the relaxation induced by ME-leaves, two protocols were carried out in aortic rings with functional endothelium. In the first protocol to investigate the effects of ME-leaves on the contraction induced by intracellular calcium release, Ca²⁺-free Krebs's solution, was replaced the regular Krebs's solution. The rings were allowed to stabilize for 15 min, followed by incubation of ME-leaves (300 μg/mL) and subsequent addition of PE (1 μM). In the control group PE (1 μM)-induced contraction was assessed in the absence of ME-leaves. To study if extracellular Ca²⁺ influx was involved in the ME-leaves induced relaxation, the regular Krebs's solution was replaced by depolarizing Ca²⁺-free Krebs's solution. The aortic rings were allowed to stabilize for 30 min, and under this condition were incubated with ME-leaves (300 μg/mL) for 30 min. A new contraction was induced by PE (1 μM) and cumulative concentration–response curves for calcium chloride (CaCl₂, 10 μM–100 mM) were constructed. In the control group, the addition of CaCl₂ was made in the absence of ME-leaves.

Changes in vascular reactivity responses of the aorta from SHR

Aortic rings from SHR were exposed to PE (1 μM) and the cumulative concentration of ME-leaves (0.1–300 μg/mL) was added to the baths. Involvement of NO/cGMP and

calcium movement were also studied using the same protocols as described in Sects. 3.2 and 3.3.

Statistical analysis

The results were expressed as mean \pm SEM, of six or eight experiments. The data were analyzed by ordinary one-way analysis of variance (ANOVA) followed by Dunnett's post hoc test, 2-way ANOVA followed by Bonferroni's multiple comparisons, or multiple *t* test, when applicable. A value of $p < 0.05$ was accepted as statistically significant. Graphs were drawn and statistical analyses were performed using GraphPad Prism version 6.0 g (GraphPad Software, La Jolla, CA, USA).

Results

Phytochemical screening

The qualitative phytochemical screening demonstrated the presence of flavonoids and tannins as summarized in Table 1. Among the phytochemicals, tannins were detected in high concentrations in both extracts.

Total phenolic and flavonoids content

ME-leaves and-stems displayed a significant quantitative phenolic and flavonoids compounds profile. However, ME-leaves presented a higher concentration of flavonoids than ME-stems, evidencing high levels of these constituents in *E. mattosii* preparations (Table 2).

Antioxidant properties of ME-leaves and stems

As shown in Table 3, ME-leaves and stems also possess the ability to scavenge free-radical DPPH. Ascorbic acid used as a positive control (AA; 50 μ g/mL), decreases the absorption

Table 1 Phytochemical screening tests for methanolic extracts of *E. mattosii* stems and leaves

Phytoconstituents	ME-stems	ME-leaves
Alkaloids	–	–
Antraquinones	–	–
Coumarins	–	–
Flavonoids	+	+
Saponins	–	–
Steroids/terpenes	–	–
Tannins	+++	++

+++ : High concentration, ++ : medium concentration, + : low concentration and – : absence

Table 2 Total phenolic and flavonoids contents of ME-leaves and stems

<i>E. mattosii</i> (μ g/mL)	TAE \pm S.E.M		QE \pm S.E.M	
	ME-stems	ME-leaves	ME-stems	ME-leaves
50	1824 \pm 8.4	1042 \pm 3.0	1.58 \pm 0.03	4.33 \pm 0.08
100	2670 \pm 15.0	2618 \pm 322.0	1.89 \pm 0.02	7.16 \pm 0.06
150	3554 \pm 44.0	3671 \pm 113.9	1.97 \pm 0.01	9.32 \pm 0.12
200	5441 \pm 24.9	4168 \pm 80.6	2.55 \pm 0.05	11.16 \pm 0.27

The results are expressed as mean \pm S.E.M. of tannic acid equivalents (TAE) and quercitrin equivalents (QE) in μ g/mL

of DPPH solution around 62%. ME-leaves and stems, at 1, 10 and 100 μ g/mL, also significantly reduced the absorption of DPPH solution when compared to the control group (\approx 80–90%).

Concentration-dependent relaxation of ME and fractions of *E. mattosii* on isolated rat aorta rings in the presence and the absence of functional endothelium

The ME of stems and leaves relaxed PE-pre-contracted aortic rings in a concentration-dependent manner. The maximal relaxant effect of ME-stems was 88.3 \pm 8.1% at a concentration of 500 μ g/mL, and 83.3 \pm 4.8% to ME-leaves at a concentration of 500 μ g/mL, which was reduced to 37.2 \pm 8.7% and 42.6 \pm 7.3%, respectively, in endothelium denuded-aortic rings (Fig. 2a). Both fractions obtained from leaves (CLF-leaves and AEF-leaves) were more effective in promoting relaxation than the fractions obtained from stems (CLF-stems and AEF-stems) (Fig. 2c and b, respectively). As shown in Fig. 2b, we can visualize only the relaxation induced by AEF-stems on aorta endothelium preserved, the addition of the other fractions did not promote any relaxation.

Table 3 Effect of ME-leaves and stems on ability to scavenge the free-radical DPPH

<i>E. mattosii</i> (μ g/mL)	DPPH (μ M)	
	ME-leaves	ME-stems
Vehicle	21.35 \pm 0.35	21.35 \pm 0.35
AA	7.94 \pm 0.26 ^a	7.94 \pm 0.26 ^a
1	4.82 \pm 0.35 ^a	4.11 \pm 0.11 ^a
10	2.10 \pm 0.23 ^a	2.69 \pm 0.11 ^a
100	3.28 \pm 0.12 ^a	1.99 \pm 0.11 ^a

AA ascorbic acid (50 μ g/mL). The results are expressed as mean \pm S.E.M. Statistical comparison was performed using one-way analysis of variance (ANOVA) followed by Bonferroni's test

^aIndicates $p < 0.001$ when compared to the vehicle group

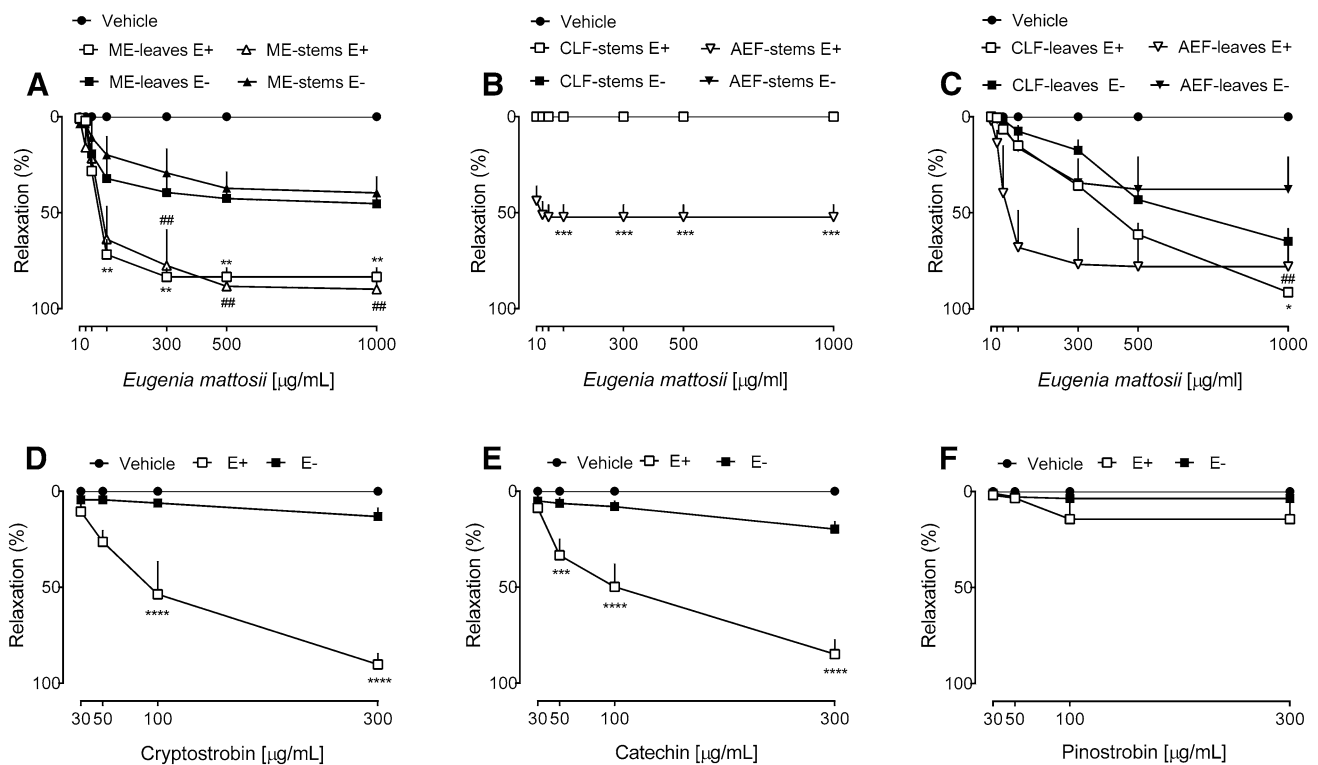


Fig. 2 Relaxation of rat aortic rings previously contracted with phenylephrine induced by methanolic extracts, fractions and compounds of *E. mattsosii*. **a** Methanolic extracts of stems and leaves; **b** chloroform (CLF) and ethyl acetate (AEF) fractions of stems and **c** CLF and AEF fractions of leaves of *E. mattsosii*. **b, e** cryptostrobin and catechin induced endothelium-dependent relaxation. **c** Absence of vasorelaxation with pinostrobin responses obtained in endothelium-intact, as

indicated by E+ and endothelium-denuded, indicated by E-. Results are expressed as mean \pm S.E.M. of 6–8 experiments. Statistical comparisons were performed using 2-way analysis of variance (ANOVA) followed by *t* test subjected to the Bonferroni's correction. *Indicates $p < 0.05$, **indicates $p < 0.01$, ***indicates $p < 0.001$, when compared with the respective control E-, ##indicates $p < 0.01$ when compared with the respective control E-

Indeed, as we could verify, the relaxation elicited by ME-leaves at a concentration of 300 $\mu\text{g/mL}$, were quite more effective than the relaxation induced by both the fractions of leaves and stems (Table 4).

To investigate if the extract, its fractions or the isolated compounds have a potentially deleterious effect on vascular functionality, the baths were washed and allowed to rest for 60 min, and the contraction induced by PE and relaxation induced by ACh was measured again. In all experiments, Krebs's solution was used as a vehicle group. Importantly, neither extracts nor compounds tested impaired the ability of PE-induced contraction and the relaxation induced by ACh (data not shown), thus reducing the possibility of the local toxic effect, inactivation of the site of action or irreversibility of the effects.

Relaxation of aorta rings from normotensive rats by compounds isolated of *E. mattsosii*

Three phenolic compounds isolated from *E. mattsosii* were studied. In preparations with preserved endothelium, cryptostrobin and catechin induced concentration–response

relaxation. Both of them relaxed about 85% of the contraction induced by PE (1 μM), at a concentration of 300 $\mu\text{g/mL}$ (Fig. 2d and e, respectively). On the other hand, pinostrobin did not induce relaxation (Fig. 2f).

Mechanisms underlying the vascular relaxation induced by ME-leaves in endothelium intact aortic rings from normotensive rats

As aforementioned the ME-leaves relaxed rat aortic rings more effectively (ME-leaves relaxed aortic rings $\approx 83\%$) when compared to the effect demonstrated with the other preparations at the concentration of 300 $\mu\text{g/mL}$. Taking it account, we investigated the mechanisms underlying the relaxation using the ME-leaves. To investigate whether NO/cGMP pathway was involved in the ME-leaves-induced aorta relaxation, L-NAME (100 μM , an inhibitor of NO synthase) and methylene blue (MB, 100 μM), an inhibitor of soluble guanylyl cyclase (sGC) for catalyzing the formation of cGMP were added to the baths 30 min before the addition of PE (1 μM) to induce contraction. In the L-NAME and MB pretreated aortic rings, the E_{max} values were reduced to

Table 4 Vascular relaxation induced by methanolic extracts, fractions obtained from of leaves and stems and phenolic compounds of *E. mattosii* in the aorta pre-contracted by PE (1 μ M)

<i>E. mattosii</i>	E_{\max} (300 μ g/mL)	
	$E+$	$E-$
ME-leaves	83.4 \pm 4.7 ^{aaabbb}	39.4 \pm 8.1 ^{bb}
ME-stems	77.5 \pm 18.8 ^{aaabbb}	29.2 \pm 12.7
CLF-leaves	35.8 \pm 8.6 ^{aaabbb}	17.4 \pm 5.5 ^b
CLF-stems	0.0	0.0
AEF-leaves	76.8 \pm 8.4 ^{aaabbb}	34.3 \pm 6.2 ^{bbb}
AEF-stems	52.3 \pm 6.6 ^{aaabbb}	0.0
Cryptostrobin	90.1 \pm 5.8 ^{aaabbb}	13.3 \pm 4.7
(-)Catechin	84.8 \pm 7.6 ^{aaabbb}	19.6 \pm 4.2 ^{bb}
Avicularin	18.7 \pm 2.8 ^{aaab}	8.0 \pm 4.0 ^b
Pinostrobin	14.4 \pm 8.8 ^{ab}	3.6 \pm 2.6

Values are presented as mean \pm S.E.M. Statistical comparisons were performed using 2-way analysis of variance (ANOVA) followed by *t* test subjected to the Bonferroni's correction

Methanolic extract of leaves (ME-leaves), Methanolic extract of stems (ME-stems), Chloroform fraction of leaves (CLF-leaves), Chloroform fraction of stems (CLF-stems), ethyl acetate fraction of leaves (AEF-leaves), ethyl acetate fraction of stems (AEF-stems)

^aIndicates $p < 0.05$, ^{aa}indicates $p < 0.01$ and ^{aaa}Indicates $p < 0.001$ when compared to the respective control vessels without functional endothelium

^bIndicates $p < 0.05$, ^{bb}indicates $p < 0.01$ and ^{bbb}Indicates $p < 0.001$ when compared to the maximal contraction obtained before addition of any fraction

14.5 \pm 2.4% and 40 \pm 7.3%, respectively (Fig. 3a, b, respectively). The incubation of atropine (1 μ M, a muscarinic receptor antagonist) and indomethacin (10 μ M, an inhibitor of cyclooxygenase) did not change the ME-leaves induced relaxation (Fig. 3c, d, respectively).

In SHR aortic rings pre-contracted with PE (1 μ M) with preserved endothelium, ME-leaves also induced a concentration-dependent relaxation, just like in normotensive rats (64.2 \pm 4.7% and 83.3 \pm 4.8%, respectively), (Fig. 4a). As shown in Fig. 4b, pre-incubation with L-NAME significantly reduced the relaxing effect of ME-leaves (from 64.2 \pm 4.7 to 14 \pm 3.6% $p < 0.0001$). MB did not modify the maximal response, but the relaxation was reduced at 50 μ g/mL (35.8 \pm 7.2 to 15.4 \pm 2.6%) and at 100 μ g/mL (55.0 \pm 8.9 to 28.1 \pm 2.3%) (Fig. 4c).

Involvement of ME-leaves (300 μ g/mL) on extracellular Ca^{2+} influx and intracellular Ca^{2+} release

As shown in Fig. 5a, in the Ca^{2+} free-Kreb's solution, PE (1 μ M) induces a transient contraction due to the release of intracellular Ca^{2+} . The second contraction induced by PE (1 μ M) in the presence of ME-leaves (30 min), was

significantly decreased (from 0.71 \pm 0.21 to 0.44 \pm 0.06 g, $p < 0.05$). To evaluate the effect of ME-leaves on calcium influx the aortic rings were exposed to PE (1 μ M) in preparations kept in depolarizing Ca^{2+} -free solution, which produced a small increase in the resting tone, and subsequent cumulative concentration–response curves for CaCl_2 (10 μ M–100 mM) was constructed. However, when the aortic rings were pre-incubated with ME-leaves (300 μ g/mL) for 30 min before PE was applied, the maximal contraction induced by cumulative concentration–response curves for CaCl_2 was reduced (from 1.53 \pm 0.15 to 1.05 \pm 0.11 g; $p < 0.05$) (Fig. 5b).

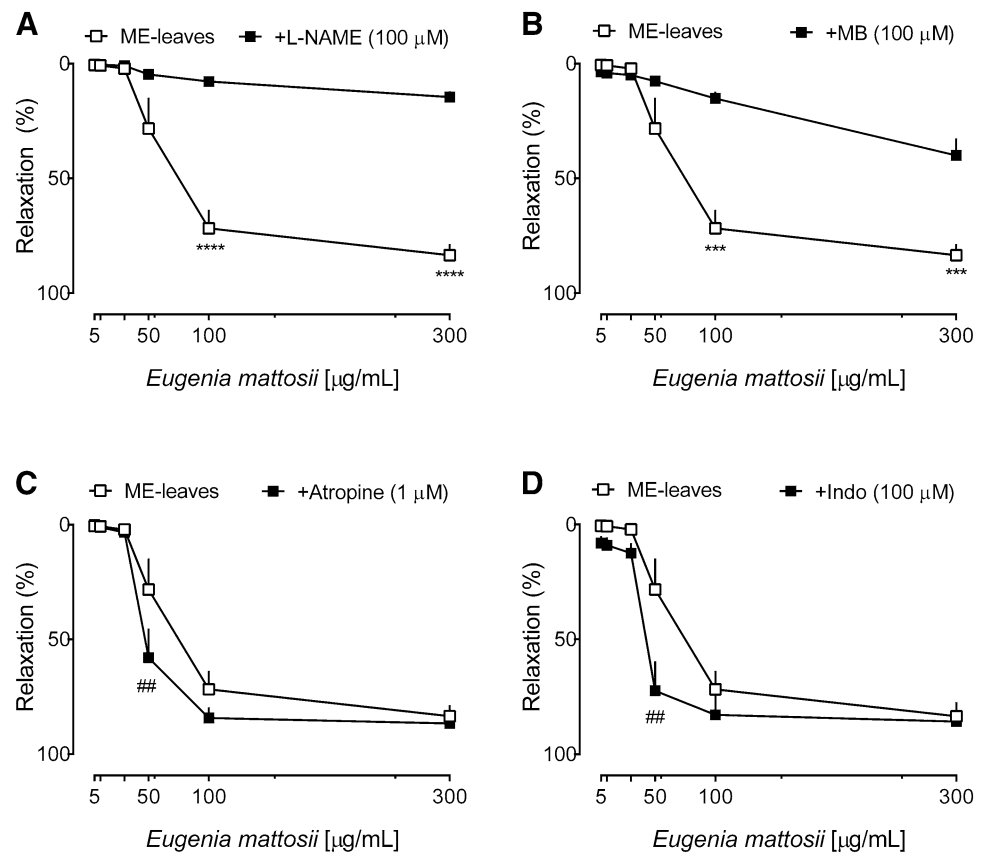
Calcium movement was also studied in aortic rings from SHR, and the same protocols described above with NTR aortic rings were used. In Ca^{2+} -free Kreb's solution preparations, PE (1 μ M) induced a reduced contraction in aortic rings from SHR (0.32 \pm 0.09 g) (Fig. 5c), compared to those of NTR (0.71 \pm 0.21 g) (Fig. 5a). However, pre-incubation with ME-leaves (300 μ g/mL) in aortas from SHR, did not modify the maximal contraction induced by PE (Fig. 5c). Cumulative addition of CaCl_2 (10 μ M to 100 mM), in aorta from SHR, in the presence of ME-leaves, was significantly reduced (Fig. 5d).

Discussion

Our study provides that methanolic extracts of leaves and stems of *E. mattosii* induced a concentration-dependent relaxation on aortic rings pre-contracted with PE. ME-leaves and stems of *E. mattosii* were able to relax both endothelium-intact and denuded aortic rings. However, the relaxation in the aorta with endothelium was greater than in aorta without endothelium, suggesting that the relaxation effect was mainly mediated through an endothelium-dependent mechanism. Although additional experiments were not carried in our current study, ME-leaves may contain compounds that act synergistically inducing vasodilation, since the extract was more effective than the chloroform and ethyl acetate fractions in the concentration of 300 μ g/mL. Therefore, the extract components could act at different targets enhancing the vasodilatation activity. Both phenolic compounds isolated from ME-leaves, cryptostrobin from the chloroform fraction, and catechin from the ethyl acetate fraction induced concentration-dependent relaxation in PE-contracted aortic rings from normotensive rats. Many studies showed that catechin has a vasodilatory effect on vascular and non-vascular smooth muscle.

It is well described that phenolic compounds, which were quantified in ME-leaves and stems, could show antioxidant activity (Oroian and Escriche 2015). Indeed, in our experiments both ME-leaves and stems showed a high profile of phenolic contents, as well as a significant DPPH radical

Fig. 3 Effect of enzyme blockers and antagonists on endothelium-dependent relaxation induced by ME-leaves in aortic rings with functional endothelium. Blockage of endothelium-dependent relaxation induced by ME-leaves by the non-selective nitric oxide synthase inhibitor L-NAME (a), and the soluble guanylate cyclase inhibitor methylene blue (b); c, d lack of influence of membrane muscarinic receptor antagonist atropine and the cyclooxygenase inhibitor indomethacin, against ME-leaves-induced rat aortic relaxation. Results are expressed as mean \pm S.E.M. of 6–8 experiments. Statistical comparisons were performed using 2-way analysis of variance (ANOVA) followed by *t*-test subjected to the Bonferroni's correction. ***Indicates $p < 0.001$, when compared with the respective vehicle



scavenging-linked antioxidant activity. It is well described in the literature that a high intake of polyphenol rich-diet from natural products may improve cardiovascular health (Sofi et al. 2014; Basu et al. 2010). Several studies confirmed that polyphenol-rich sources such as extracts from red wines, green and black tea, and several plants caused endothelium-dependent relaxations in large arteries, arterioles, and veins that were prevented by competitive inhibitors of the enzymes endothelial nitric oxide synthase and guanylyl cyclase (Fitzpatrick et al. 1993; Schini-Kerth et al. 2010).

Considering the vasodilator effects obtained with ME-leaves, we herein studied its mechanism of relaxation in thoracic aorta strips with functional endothelium. The vascular endothelium plays a central role in the control of vascular tone. The monolayer of endothelial cells is located between the vascular lumen and the smooth muscle cells of the vessel wall and can release vasoactive substances from endogenous ligands such as acetylcholine, bradykinin, histamine, and by endogenous blood flow as, shear stress. Through these stimuli, the endothelium cells produce vasodilators such as endothelium-derived relaxing factor (EDRF), endothelium-derived hyperpolarizing factor (EDHF) and prostacyclin (Furchgott and Zawadzki 1980; Feletou and Vanhoutte 1988; Moncada and Vane 1978), with these findings, the vascular endothelium emerged as an important regulator of vascular tone and blood pressure. Among them, the most important

and studied vasodilators seem to be the EDRF which has been identified as the nitric oxide (NO) radical (Ganz and Ganz 2001). NO is produced after activation of endothelium nitric oxide synthase (eNOS also called NOS III) by acetylcholine (ACh), and results in synthesis and release of NO, which in turn leads to guanylate cyclase activation and cyclic guanosine 3',5-monophosphate (cGMP) elevation, leading to vascular smooth muscle relaxation, for review see Moncada and Higgs (2006). Therefore, based on the inhibition of ME-leaves-induced relaxation by L-NAME, it implies that ME-leaves induce the release of NO from endothelium cells.

Apart from NO, PGI₂ is another important endothelium-derived relaxation factor, so the effect of ME-leaves on PGI₂-mediated vascular dilation was also studied. Prostaglandins are synthesized by cyclooxygenase (COX) enzymes. The relaxing effect of ME-leaves was not inhibited by indomethacin (a non-selective inhibitor of COX), as well as was not affected by the pretreatment with atropine (a muscarinic receptor antagonist), suggesting ME-leaves-induced vasorelaxation neither involves the prostaglandin pathway nor activation of endothelial muscarinic receptor (type M₃). However, as we could observe after the inhibition of COX with indomethacin, there was an intensification of the relaxation. These results suggest that endothelial COX when not inhibited should contribute to the production of a contractile factor and that this factor has been lost after its

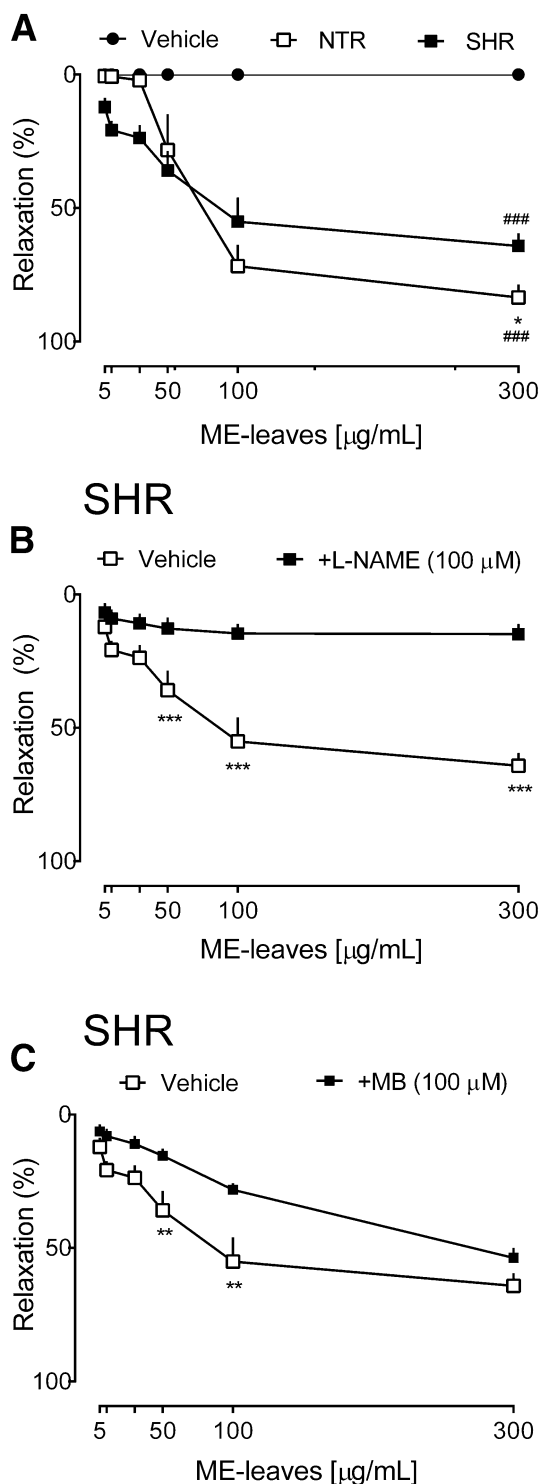


Fig. 4 Assessment of the vasodilator effect of ME-leaves in aortic rings of normotensive (NTR) and hypertensive (SHR) rats. Vascular relaxation induced by ME-leaves in aortic rings of NTR and SHR, responses obtained in endothelium-intact vessels (a). Blockage of endothelium-dependent relaxation induced by ME-leaves by the non-selective nitric oxide synthase inhibitor L-NAME in aortic rings of SHR (b) and, c reduction of the relaxation promoted by addition of methylene blue in aortic rings of SHR. The results show the mean \pm S.E.M. of six experiments. Statistical comparisons were performed using 2-way analysis of variance (ANOVA) followed by *t*-test subjected to the Bonferroni's correction. **Indicates $p < 0.01$, ***indicates $p < 0.001$ when compared to vehicle

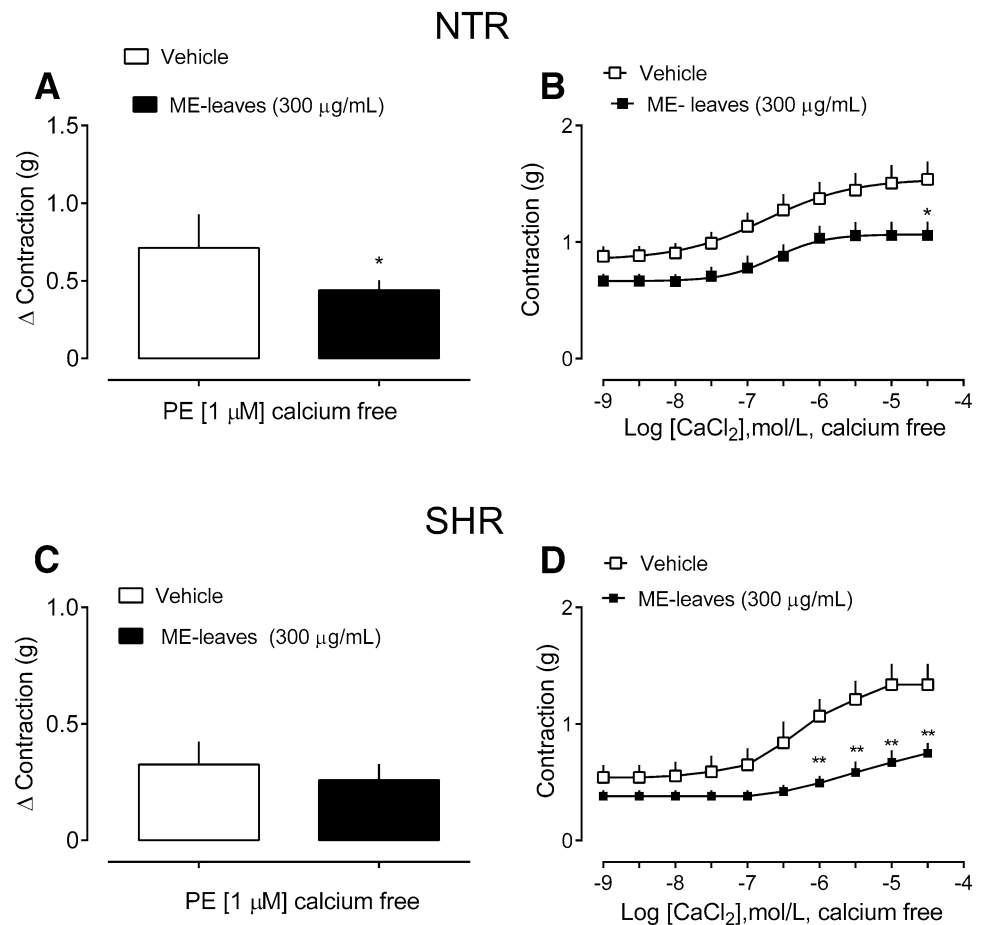
inhibition, or that COX after its inhibition contributes to the potentiating in the production of a relaxing factor.

The depletion of intracellular calcium significantly reduced the contraction induced by PE in preparations previously incubated with ME-leaves, as well as reduces the maximal contraction obtained in response to the cumulative addition of Ca^{2+} in depolarizing Krebs's solution. However, in regular Krebs's solution, ME-leaves pre-incubation did not affect PE-induced contraction but was able to significantly reduce the contraction induced by KCl. Calcium movement plays an important role in the NO synthesis and releases in endothelium cells, contributing to endothelium-dependent relaxation (Loeb et al. 1988; Fleming and Busse 1999). Otherwise, the contraction of smooth muscle is also regulated by cytosolic Ca^{2+} level and the sensitivity to Ca^{2+} of the contractile elements in response to changes in the environment surrounding the cell (Karaki et al. 1997). Studies show that lowering intracellular calcium ($[\text{Ca}^{2+}]_i$) reduced ACh-induced relaxation emphasizing the importance of extracellular Ca^{2+} in endothelium-dependent relaxation (Long and Stone 1985; Hayashi and Hester 2010), and it seems likely that $[\text{Ca}^{2+}]_o$ plays an important role not only in contractile processes but also in relaxant processes as well (Godfraind et al. 1986). In the same way, contraction induced by KCl was reduced in the presence of ME-leaves. The cellular mechanism of contraction involved in response to KCl is that a high concentration of KCl causes a membrane depolarization, which leads to an increased Ca^{2+} influx through voltage-dependent calcium channels (VDCCs). Our data suggest that Ca^{2+} may be involved in the ME-leaves induced vasorelaxation.

We wondered if the aorta from hypertensive responds differently from the normotensive. Hypertension in SHR is associated with smooth muscle hypertrophy and changes in the structure and composition of the arterial wall (Levy et al. 1994), which leads to arterial rigidity and deficiency of the smooth muscle reduce the aortic compliance. Such changes when occurring in resistance vessels may lead to the change of peripheral resistance, and consequently hypertension (Wright and Angus 1999; Intengan and Schiffrin 2001). In our study, we found that aortas of hypertensive rats presented a reduction in acetylcholine-induced vasodilator response and a reduction of vasoconstrictor response to phenylephrine and KCl (data not shown). Differences in maximal responses between SHR and NTR tissues for contracting substances (PE and KCl) can be attributed to reduced smooth muscle contractility and/or changes in the mechanical properties of the isolated vessel.

Hypertension is a chronic and multifactorial disease and is a major risk factor for other severe cardiovascular conditions (Vanhoutte 1996). Hypertension has been associated with changes in vascular endothelium production and the release of vasoactive substances and is related to endothelial

Fig. 5 Effects of ME-leaves on contraction induced by phenylephrine and calcium in aortic rings with functional endothelium of NTR and SHR. NTR (a) and SHR (c), Phenylephrine-induced contraction of vessels maintained in calcium-free PSS in the absence or after incubation of ME-leaves. NTR (b) and SHR (d) Calcium-induced contraction in the absence or after incubation of ME-leaves in depolarizing Krebs solution. The results show the mean \pm S.E.M. of 6–8 experiments. Statistical comparisons were performed using 2-way analysis of variance (ANOVA) followed by *t* test subjected to the Bonferroni's correction, or Student's *t* test when applicable (a, c and d). *Indicates $p < 0.05$, ***indicates $p < 0.001$ when compared to vehicle



dysfunction and changes in vascular smooth muscle reactivity (Lunardi et al. 2009), both in animals and human models.

Spontaneously hypertensive rats (SHR) are useful for studying the mechanism involved in the endothelium dysfunction conditioned by hypertensive vasoconstriction (Pinto et al. 1998). Hypertension in SHR animals is like essential hypertension in humans (Gendron et al. 2004), in this way has become an experimental tool widely used for the study of hypertension in pre-clinical trials. Some studies demonstrated that aorta from SHR shown reduced ACh-induced vascular endothelium-dependent relaxation. This impairment is considered to be due to a decreased release of NO, a decreased release of EDHF, or an increased release of endothelium-dependent contraction factor (EDCF) (Diederich et al. 1990; Fujii et al. 1992; Hayakawa et al. 1993), and reduced contractility response to vasoconstrictor, in other words, vasoconstrictor are weaker in the aorta of SHR than in those from normotensive rats (Gendron et al. 2004). In SHR, in which hypertension develops gradually over weeks, alterations in aortic wall properties precede the development of hypertension, such vascular lesions should, therefore, be considered as a cause and not a consequence of high blood pressure (van Gorp et al. 2000; Gendron et al. 2004).

Moreover, hypertension is associated with endothelium dysfunction, which is mainly defined by decreased vasodilator potential (Spijkers et al. 2010; Intengan and Schiffrin 2001). Thus, based on the participation of the NO/cGMP pathway in the vasorelaxation effect of ME-leaves, it was interesting to investigate if ME-leaves could induce vasorelaxation in aorta from SHR.

In our study, we found that the ME-leaves of *E. mattosii* was able to induce relaxation in aortic rings SHR pre-contracted with PE, in the same way as in normotensive rats, L-NAME, but not MB, completely inhibited ME-leaves-induced aorta relaxation. As already described above sGC present in vascular smooth muscle is the major target of NO to induce relaxation. The difference in relaxation inhibition is probably because L-NAME acts on NOS activity, inhibiting NO synthesis and production. Whereas, MB blocks the activation of guanylate cyclase by NO. In the presence of ME, NO is continuously being produced and diffuses into the vascular smooth muscle. NO is known to promote muscle relaxation at least in part through the activation of K⁺ channels (Faraci and Heistad 1998; Nelson and Quayle 1995). Opening a K⁺ channel present in the vascular smooth muscle membrane increases K⁺ efflux into the extracellular

environment, leading to hyperpolarization (Sobey 2001). Hyperpolarization, in turn, leads to the closure of voltage-dependent calcium channels, reducing calcium intake and consequently inhibiting contraction (Nelson and Quayle 1995). Potassium channels also play an important role in vascular relaxation through vascular smooth muscle polarization. Probably it is through this mechanism that we still see some relaxation with methylene blue. Furthermore, they are direct or indirect targets of the actions of NO in the vasculature (Sobey 2001; Costa and Assreuy 2005). The production of NO by the ME-leaves may be modulating potassium channels and thus inducing vasodilation. As in normotensive, the presence of the extract reduced the contraction induced by the cumulative addition of CaCl₂ in the aorta of SHR. Thus, it is reasonable to state that the relaxant effect of ME-leaves can be related to activation of NO/cGMP pathway and at least in part to some action on extracellular calcium uptake or changes in calcium mobilization from intracellular stores.

Taken together our study demonstrated that *E. mattosii* ME-leaves, fractions and isolated phenolic compounds were able to induce relaxation in aortic rings from normotensive and hypertensive rats. The major mechanism by which ME-leaves induce relaxation involves the NO/cGMP pathway, however, other studies are necessary to clarify the other possible mechanism of action, including those endothelium-independent. The pharmacological findings described in this study may be an important step for validation of the popular usage of *E. mattosii* as a phytomedicine for the treatment of vascular diseases. Our findings may constitute the basis for further functional studies, which is going to reveal the mechanism by which cryptostrobin and catechin may induce vascular relaxation. Besides, both the efficacy and safety for acute and chronic usage of preparations obtained from *E. mattosii* remains to be investigated. Indeed, the pharmacological treatment of cardiovascular diseases such as hypertension includes flavonoid-rich diets. Phenolic compounds are mostly ascribed to their antioxidant and vasodilator actions (Zenebe et al. 2001; Nijveldt et al. 2001; van den Elsen et al. 2014). Also, as already mentioned above, previous phytochemical studies have revealed the presence of steroids, terpenes and phenolic compounds as the main constituents from *E. mattosii*.

Conclusions

In conclusion, our study has demonstrated that ME-leaves from *E. mattosii* can induce a concentration-dependent relaxation on both endothelium-intact and denuded thoracic aortic rings pre-contracted by PE from normotensive and spontaneously hypertensive rats. The endothelium-dependent relaxation may be predominantly mediated by the NO/cGMP pathway. Furthermore, extracellular calcium uptake

or changes in calcium mobilization from intracellular stores. The phenolic compounds cryptostrobin and catechin also induced endothelium-dependent relaxation an event that might also involve NO pathway. Moreover, cryptostrobin and catechin induced relaxation, which may contribute synergistically to the vasorelaxation effect of the ME-leaves. The finding we have presented suggest that *E. mattosii* has the potential for further studies in animal models of cardiovascular diseases.

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Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

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